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Rickettsia sennetsu in Human Blood Monocyte Cultures: Similarities to the Growth Cycle of *Ehrlichia canis*

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Microscopic examination of cultured human monocytes infected with *Rickettsia sennetsu* and stained by the Giemsa method revealed the presence of various organismal growth forms in the cytoplasm of the infected cells. The growth forms were loosely scattered individual organisms, clusters of organisms, various sizes of dense inclusion bodies in intact and vaculated cytoplasm, and organisms in close proximity to disintegrated monocytes. The appearance and the morphology of these *R. sennetsu* growth forms were similar to those of *Ehrlichia canis* propagated in canine monocytes. Specific identification of *R. sennetsu* was made by staining cultured monocytes with fluorescein-conjugated globulins extracted from the pooled sera of patients recovering from sennetsu rickettsiosis. Mice infected with cultured *R. sennetsu* developed gross pathological changes indicative of infection, and the organism was demonstrated in their spleens, peritoneal macrophages, and mononuclear blood cells. Human monocyte culture appeared to be more sensitive than the previously used African green monkey kidney cell line (BSC-1) for the isolation of *R. sennetsu* from samples containing minute infectious quantities of this organism.

Rickettsia sennetsu is the causative agent of sennetsu rickettsiosis in western Japan. The disease is characterized by fever, malaise and headache, lumbago, anorexia, sleeplessness, drenching sweat, constipation, and lymphadenopathy (8). The means of transmission has not been established, although arthropods are suspected. Since the biological properties of *R. sennetsu* are not well understood, the organism is regarded as a species *incertae sedis* (14).

Ehrlichia canis is the causative agent of canine ehrlichiosis, which is manifested by progressive pancytopenia, particularly thrombocytopenia; anorexia; emaciation; dehydration; and increased body temperature. A fulminating form of the disease, manifested by epistaxis, which caused severe losses among military dogs in Viet Nam during 1968 to 1970, is referred to as tropical canine pancytopenia (5). The disease is transmitted by the tick *Rhipicephalus sanguineus*. In naturally infected hosts, both *R. sennetsu* and *E. canis* are found in the cytoplasm of peripheral blood monocytes.

Although *R. sennetsu* exhibits certain properties of rickettsiae, no serological relationship to other rickettsiae has been reported. Similarly, no antigenic relationship was established between *E. canis* and other rickettsiae (10). However, very recently, the sera of patients recovering from sennetsu rickettsiosis revealed cross-reactivity with *E. canis* in an indirect fluorescent-antibody test (unpublished data).

In addition to an antigenic relationship, *R. sennetsu* and *E. canis* appear to be morphologically similar. Unlike most other rickettsiae, these two agents do not occur freely in the cytoplasm of infected cells. Rather, single or multiple forms of the organism are contained in a membrane-lined vacuole (1).

Because of these serological and morphological resemblances between *R. sennetsu* and *E. canis*, the antigenic uniqueness of the two organisms with reference to other pathogenic rickettsiae, and our experience with the propagation of *E. canis* in blood monocytes, growth of *R. sennetsu* in human blood monocyte cultures was attempted.

MATERIALS AND METHODS

Organisms. *R. sennetsu* was isolated from a patient by Misao and Kobayashi in 1953 (8) and has been referred to as the Miyayama strain. The agent was purchased from the American Type Culture Collection (ATCC) and was received frozen in dry ice. The organism on deposit in ATCC had been propagated in green monkey kidney cell line BSC-1.

E. canis was recovered in Florida in 1969 from the blood of a German shepherd dog with signs of canine

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ehrlichiosis and was propagated in canine blood monocyte cultures (9).

Cell cultures. Two cell cultures were used to isolate and propagate *R. sennetsu* received from ATCC: African green monkey kidney cells and human blood monocyte cultures.

The African green monkey kidney (BSC-1) cell line was purchased from Microbiological Associates. The BSC-1 cells were grown in Eagle minimal essential medium with Earle salts, supplemented with 2 to 10% fetal bovine serum and 1% L-glutamine. No antibiotics were used in the medium. Cell monolayers were propagated in 25-cm^2 plastic tissue culture flasks and Leighton culture tubes, each provided with cover slips (0.9 by 4.0 cm). The cells in culture reached 70 to 80% confluency in 3 to 4 days of incubation at 37°C and were ready for use at that time.

The second type of cell culture consisted of human blood monocytes. Blood (40-ml volume) was obtained from the cephalic arm veins of research team volunteers at the University of Illinois infirmary. Each blood sample was collected in a 50-ml sterile syringe coated with heparin (160 U). A 10-ml amount of a 0.25% dextran sulfate (molecular weight, 500,000) solution in normal saline warmed to room temperature was aseptically added to the blood in the syringe. The syringe was placed in a vertical position (needle end up), and erythrocytes were allowed to sediment for 60 min at 22°C. The needle was then bent, and 5-ml volumes of the plasma phase were infused into 25-cm^2 tissue culture flasks. The flasks were placed in a 37°C incubator, and monocytes were allowed to attach during a period of 3 to 5 h. The supernatant was then poured off, and 5 ml of medium consisting of Eagle minimal essential medium with Earle salts, supplemented with 20% human serum and 1% L-gluatmine (400 mM), was added. After 24 h of incubation, the medium was replaced with fresh medium and every 2 to 3 days thereafter or when the medium turned alkaline. Within 2 weeks of incubation, monocytes increased considerably in size and formed a solid monolayer.

Propagation of *R. sennetsu*. *R. sennetsu* was thawed in a 37°C water bath and rapidly diluted 1:5 with the above-mentioned medium supplemented with 10% fetal calf serum for infection of the BSC-1 cell culture. Growth medium was removed from the BSC-1 cells before the addition of the organism. Cell monolayers in flasks and Leighton tubes were inoculated with 2 or 0.5 ml of the diluted material and allowed to stand for 2 h for adsorption of the organism. The medium containing 10% fetal calf serum was then added, and the cultures were maintained at 37°C. The serum concentration was reduced to 2% fetal calf serum when the cells reached a confluent monolayer.

For inoculation of blood monocyte cell cultures, the material was thawed by rapid immersion in a 37°C water bath and diluted 1:5 with Eagle minimal essential medium with Earle salts, supplemented with 10% human serum. The medium was removed from the monocyte culture flasks before the addition of *R. sennetsu*.

The time allowed for adsorption of the rickettsiae was 2 h at 37°C, after which 3 ml of the medium was added, and the cultures were returned to the incubator. The supernatant of the first culture was used to infect a second culture. The medium was changed every 3 to 4 days or whenever the supernatant became alkaline.

Examination of cultures for the presence of *R. sennetsu*. To ascertain the presence of *R. sennetsu* in the BSC-1 cells, cover slips were removed from the cultures after 6 to 20 days of cultivation. The cover slips were cut in half and stained by either the Giemsa or the direct fluorescent-antibody method. Serum from five patients recovering from sennetsu rickettsiosis were pooled, and gamma globulins were extracted and used to prepare a conjugated fluorescein isothiocyanate stain for *R. sennetsu*. A similar preparation of fluorescein isothiocyanate-conjugated gamma globulins obtained from five apparently normal human subjects was used as a control. Uninfected cell cultures served as additional controls. The methods used for extraction, purification, and conjugation of gamma globulins was described earlier (10). Cover slips were fixed in absolute acetone at 22°C for 5 min, allowed to dry for 15 min, and flooded with conjugated antiglobulins. The cover slips were placed in a humidified chamber and incubated at 37°C for 30 min; they were rinsed twice (5 min each time) in phosphate-buffered saline, pH 7.2; rinsed for 5 min in distilled water, and air dried. A drop of mounting fluid containing 9 parts of glycerin and 1 part of phosphate-buffered saline was deposited on each cover slip, and the cover slip was placed in an inverted position on a microscope slide. Permount fluid was placed along the edges of the cover slip to attach it to the slide. The slides were examined on a microscope equipped with an ultraviolet light source.

Human blood monocyte cultures were examined for *R. sennetsu* each time the medium was changed, generally every 3 or 4 days. Approximately 2.5 ml of the supernatant medium was poured into a round, 5-ml, flat-bottomed glass tube. The bottom of the tube was provided with a round cover slip. The tube was centrifuged at 750 × *g* for 15 min, the supernatant was discarded, and the cover slip was stained by the Giemsa or fluorescent-antibody technique.

Twenty-four white laboratory mice divided into two equal groups were used to ascertain the infectivity of *R. sennetsu* propagated in monocyte cultures. Each of the 12 mice in one group were inoculated intraperitoneally with 0.2 ml of the supernatant of the 11-day-old cultures. The remaining 12 mice, which served as controls, received similar supernatant fluid from *R. sennetsu*-free cultures. Four mice of each group were euthanized every 8 days after infection. In addition to gross pathological observations, slides of peritoneal macrophages, splenic cross sections, and blood were stained by the Giemsa technique and examined microscopically.

Examination of cultures for the presence of *E. canis*. Supernatant fluids from the canine monocyte culture infected with *E. canis* were collected on different days of incubation and processed for examination in the same fashion described above for *R. sennetsu*.

RESULTS

Repeated efforts to isolate *R. sennetsu* in the BSC-1 cell line from two shipments of the organism received from ATCC were unsuccessful.

The results of microscopic examination of the cultures and mouse inoculation indicated that the organism did not grow in the BSC-1 cells. An attempt to grow the organism in human blood monocytes seemed to be the next logical step.

Sequential examination of cultured human blood monocytes stained by the Giemsa technique revealed various growth forms of *R. sennetsu*. Rickettsiae were detected as early as 3 days after infection. At this stage, the cytoplasm of infected monocytes showed slight discoloration. The organisms were relatively evenly distributed throughout the cytoplasm (Fig. 1A). During the subsequent days of incubation, the number of organisms per monocyte greatly increased, nearly saturating the cytoplasmic portion of the host cell. These and other growth forms have been specifically identified by use of the direct fluorescent-antibody method. An infected monocyte stained by the fluorescent-antibody method is shown in Fig. 1B. By day 7, numerous cells containing organisms in the form of loosely packed clusters were noted (Fig. 1C). Subsequently, cells containing inclusion bodies (Fig. 2A) resembling the morulae of *E. canis* in canine monocytes were observed (Fig. 2B). These inclusion bodies were oval and occasionally pleomorphic, with individual organisms not clearly differentiated (Fig. 1D). Whether there was a distinct vacuolar membrane surrounding these bodies was difficult to determine by examination with a light microscope. A dozen or more morulae-like bodies were observed in the cytoplasm of some monocytes. These bodies were always clearly demarcated from each other. At this stage, many monocytes were three to four times their original size and contained two to three nuclei, a few of which appeared to be in the process of mitosis. During days 16 to 20 of incubation, various sizes of inclusion bodies appeared dissociated from the cytoplasm and occurred freely in cytoplasmic vacuoles (Fig. 1E). This growth phase was succeeded by the apparent disintegration of monocytes and a gradual release of the organism into the media (Fig. 1F). In most instances, organisms occurring extracellularly in the medium remained in the form of loosely packed clusters. Normal cell cultures remained free of artifacts resembling inclusion bodies or granular material that could have interfered with the identification of rickettsiae in the infected cultures.

All three groups of infected mice euthanized at 8, 16, and 24 days after infection revealed the presence of the organism in peritonial macrophages, blood, and splenic impression smears stained by the Giemsa method. Organisms were usually seen as a focal infection in the form of a nestlike arrangement occurring in the cytoplasm of splenic cells and similarly in the cytoplasm of peritoneal macrophages and mononuclear blood cells. Major gross tissue abnormalities included splenic hypertrophy and greatly enlarged mesenteric lymph nodes.

DISCUSSION

The use of peripheral blood monocyte cultures has proved to be a practical means for the isolation of *R. sennetsu* from samples stored at a low temperature (−65°C) for a prolonged period of time. Under the circumstances, it is possible that the infectivity titer of the samples was reduced below the threshold of a minimum infectious dose needed to initiate growth of the organism in the BSC-1 cell line. Since the latter cells have been used with good results to propagate *R. sennetsu*, one may speculate that blood monocytes in culture represent a more sensitive sytem than BSC-1 cells for the isolation of the agent from samples containing minute infectious quantities of the organism. This reasoning is supported by previous experiences with peripheral blood monocyte cultures for the isolation and propagation of other rickettsiae. This cultivation method was introduced for the first successful propagation of *E. canis*, the causative agent of canine ehrlichiosis (9). Since then, the method has been found useful for the isolation and propagation of *Neorickettsia helminthoeca* from the blood of infected dogs (2), *Rickettsia rickettsii* from the blood of infected rhesus monkeys (3, 4), and more recently *Rickettsia tsutsugamushi* from the blood of infected silver leaf monkeys, cynomolgus monkeys, and dogs (11).

The growth of *R. sennetsu* in human monocytes greatly resembles that of *E. canis* in canine monocytes. Inclusion bodies or morulae, characteristic of *E. canis*, were also present in cultivated human monocytes infected with *R. sennetsu*. The question of whether this morphological resemblance of growth stages between *E. canis* and *R. sennetsu* is a real reflection of their close biological relationship or whether it is merely a morphological expression forced upon the organism by a common host cell (monocyte) must await further studies.

The above accumulated evidence pointing to the usefulness of cultured blood monocytes for the isolation of various rickettsiae needs some elucidation of the underlying mechanisms which may be contributing to the phenomenon. Blood monocytes exist in a variety of physiological states. These differences are, in all probability, related to the effects of specific inducing agents operational in the host from which the monocytes have been derived. In the case of monocytes derived from the blood of an infected host, it would be logical to speculate that these are represented by the lymphokine-stimulated mac-

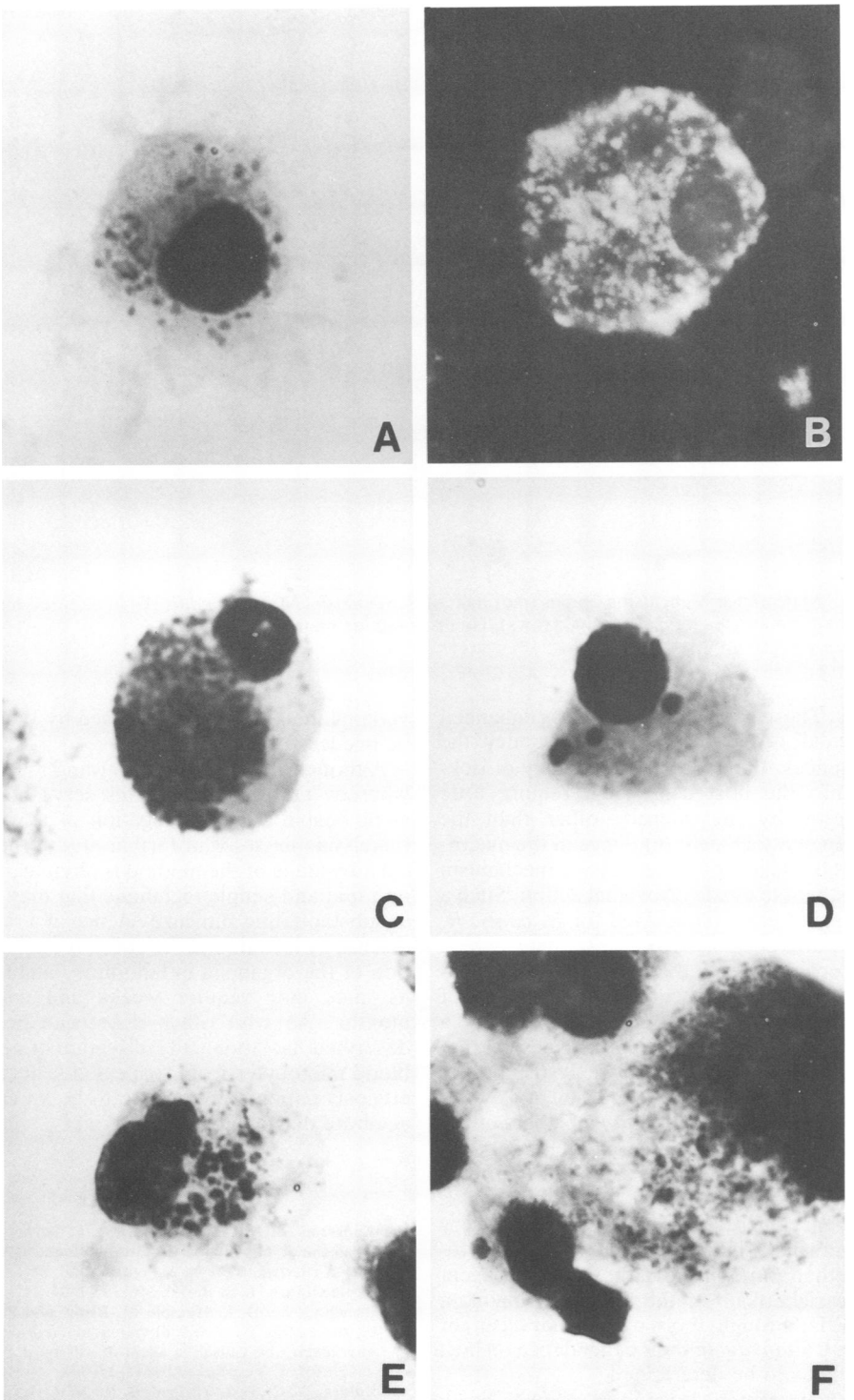

FIG. 1. *R. sennetsu* in the cytoplasm of cultured human blood monocytes. 1B was stained by the direct fluorescent-antibody method. The remaining figures were stained by the Giemsa method, ×2,100. (A) Early stage of development (3 days) with organisms loosely distributed in the cytoplasm. (B) Heavily infected monocyte on day 7 after infection. (C) Organism occurring in a loosely packed cluster after 7 days of incubation. (D) Three large inclusion bodies (morulae) at day 12 of incubation. (E) Various sizes of inclusion bodies occurring freely in cytoplasmic vacuoles at day 18 of incubation. (F) Apparently disintegrated monocyte with organisms free in the medium at day 20 of incubation.

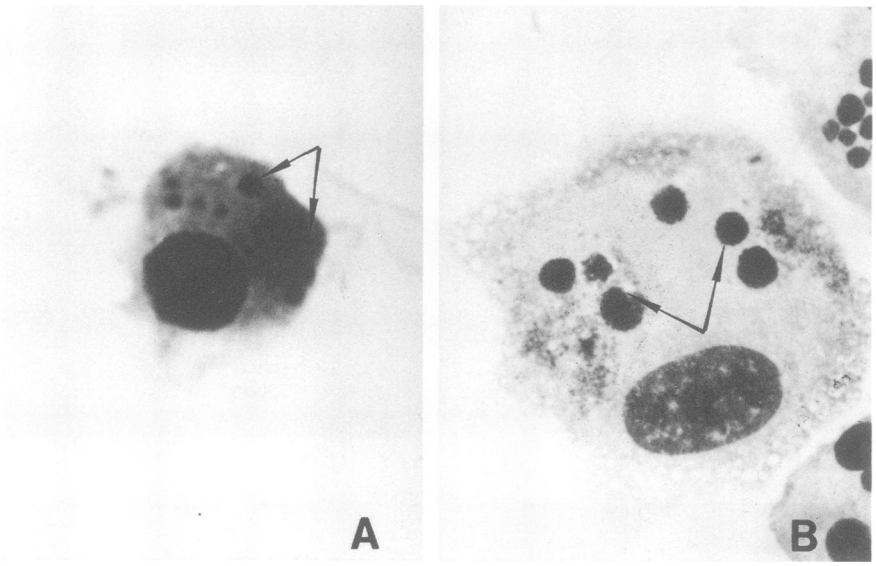

FIG. 2. Appearance of inclusion bodies (morulae) of *R. sennetsu* (A) and *E. canis* (B) in cultured human and canine blood monocytes, respectively (arrows). Giemsa staining method, ×2,100.

rophages. These macrophages possess enhanced microbicidal properties (7). Thus, under the circumstances, the process of the entry of rickettsiae into the host cell would require little participation by the microbe other than the initial step of attachment (6). Once in the macrophage, rickettsiae must possess a mechanism enabling them to evade lysosomal action. Such a mechanism is easily conceived for *E. canis*, *R. sennetsu*, and possibly *Coxiella* burnetii, which remain separated from the lysosomes and eventually multiply in the vacuoles. For members of the genus *Rickettsia*, which are deprived of a similar vacuolar barrier, Weiss suggested that they may bypass the action of the lysosomes by active penetration into the cytoplasm and avoidance of the vacuoles (13). Thus, members of the genus *Rickettsia* require energy-yielding metabolic activities to escape digestion. *Chlamydia* agents, which also have some resemblance to *R. sennetsu*, are energy parasites, but have a very efficient means of inducing their own phagocytosis. Whether *Ehrlichia* and the sennetsu agent resemble rickettsiae in the ability to maintain ATP levels through their own metabolism or resemble *Chlamydia* in their dependence on host ATP remains to be determined.

In the case of nonactivated monocytes being used to propagate rickettsiae, as is the case in the present study, one must assume that these cells possess the necessary cell surface receptors needed for the attachment and entry of these organisms (12). However, some participation by the organism in the form of energy-yielding metabolism as proposed by Weiss must be needed for its entry (13).

Although the question regarding mechanisms whereby cultured monocytes serve as an efficient host for the propagation of a variety of rickettsiae must await further study, the practical advantage of the method is obvious. There is no rapid and simple technique that may be used in substantiating clinical evidence of a rickettsial disease by isolation of the causative agent. Isolation of the organism in laboratory animals such as mice may require weeks and sometimes months. As with other rickettsiae previously described, isolation and cultivation of peripheral blood monocytes from suspected sennetsu rickettsiosis patients may prove to be an early and accurate diagnostic means.

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